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(54) Title of the Invention: Method for the detection of nucleic acids

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## SPECIFICATION

### 1. Title of the Invention

Method for the detection of nucleic acids

### 2. Scope of the Patent Claims

- 1) A method for the detection of nucleic acids, characterized in that, using a sample which contains the nucleic acid to be detected, a probe nucleic acid, and a nucleic acid for fixing purposes which has a part which bonds with a solid phase and a part which hybridises with the probe nucleic acid, there are included a) a process in which a nucleic acid to be detected - probe nucleic acid - nucleic acid for fixing purposes hybrid is formed and b) a process in which whether or not the hybrid which is bound to the solid phase has the form of a nucleic acid to be detected - probe nucleic acid hybrid is detected using a label.
- 2) A method for the detection of nucleic acids, according to claim 1, wherein the labelling is carried out on the double strand forming part of the nucleic acid to be detected and the probe nucleic acid.
- 3) A method for the detection of nucleic acids, according to claim 1, wherein the nucleic acid to be detected, the probe nucleic acid and the nucleic acid for fixing purposes are reacted simultaneously.
- 4) A method for the detection of nucleic acids, according to claim 1, wherein the nucleic acid to be detected and the probe nucleic acid are reacted and then the nucleic acid for fixing purposes is reacted with the reaction mixture so obtained.
- 5) A method for the detection of nucleic acids, according to claim 1, wherein the probe nucleic acid and the nucleic acid for fixing purposes are reacted, and then the nucleic acid to be detected is reacted with the reaction mixture obtained.

6) A method for the detection of nucleic acids, according to claim 1, in which there is a process in which the nucleic acid to be detected - probe nucleic acid hybrid is released from the nucleic acid for fixing purposes.

### 3. Detailed Description of the Invention

#### Industrial Field of Application

The invention concerns a method for the detection of nucleic acids in which nucleic acid hybrid forming reactions are used.

#### Prior Art

There are various known methods for detecting whether or not a subject nucleic acid (nucleic acid to be detected) is present in a sample.

For example, there are methods in which a labeled probe nucleic acid is reacted with a sample which has been fixed on a solid phase and whether or not a nucleic acid to be detected - probe nucleic acid hybrid has been formed on the solid phase is detected by means of the labelling which has been carried out on the probe nucleic acid, and methods in which a probe nucleic acid is reacted with a sample in the liquid phase and the presence or absence of a nucleic acid to be detected - probe nucleic acid hybrid is detected in the reaction mixture obtained.

In one example of the former method the sample is first fractionated using electrophoresis and a replica of the electrophoresis pattern of the sample is formed on a nitrocellulose filter by plotting. A radioactive probe nucleic acid is reacted with this replica. At this time a hybrid is formed between the nucleic acid to be detected and the radioactive probe nucleic acid in those cases where the nucleic acid to be detected is present in the sample. Subsequently, the unhybridized

radioactive probe nucleic acid is removed by washing, and then the radioactively labelled region of the hybrid which has been formed on the nitrocellulose filter is detected using, for example, autoradiography.

In an example of the latter method a radioactive probe nucleic acid is reacted with the sample in solution and the hybrid which is formed in those cases where the sample contains the nucleic acid to be detected and the unreacted material are separated using a column technique. Detection is achieved by measuring the radioactive isotope label activity of the hybrid which has been formed.

Furthermore, there are also methods in which the probe nucleic acid is fixed to a column, the sample is passed through and reacted and, after eluting the hybrid which has been formed, then separating the hybrid into its individual strands which are then concentrated and detected.

#### Problems to be Resolved by the Invention

With the aforementioned methods in which the sample has been fixed to a solid phase, large amounts of sample are often wasted and the detection procedure is time consuming, and there is a further disadvantage in that the preparation of the solid phase is complicated and requires many processes.

Furthermore, in the methods where a probe nucleic acid is reacted with the sample in the liquid phase and the hybrid which has been formed and the unreacted material are separated using a column technique, there is a disadvantage in that, in cases where the nucleotide chain of the nucleic acid to be detected is comparatively short, the precision of separation with a column technique is often poor.

Furthermore, with the methods in which a probe nucleic acid which has been fixed on a column is used, there is a disadvantage in that the column can be used only for a specific application, which is to say that it can only be used in cases where a specific nucleic acid base sequence is to be detected, and it cannot be used generally. Furthermore, in those cases where the detection of a plurality of nucleic acid base sequences has to be detected, there is a problem in that probe nucleic acids corresponding to the respective base sequences must be prepared and fixed on separate columns.

The present invention, the result of thorough research carried out with a view to resolving the above-mentioned problems associated with the conventional methods of detection in which a hybridisation technique is used, is intended to provide a method with which the presence of a nucleic acid as a specific subject for detection can be detected both quickly and easily.

#### Means of Resolving These Problems

The method of detection of this invention is characterized in that, using a sample which contains the nucleic acid to be detected, a probe nucleic acid, and a nucleic acid for fixing purposes which has a part which bonds with a solid phase and a part which hybridises with the probe nucleic acid, there are included a) a process in which a nucleic acid to be detected - probe nucleic acid - nucleic acid for fixing purposes hybrid is formed and b) a process in which the formation of a nucleic acid to be detected - probe nucleic acid hybrid is detected using a hybrid label which has been bound to the solid phase (sic).

The invention can be used to detect various nucleic acids such as DNA of biological origin and DNA which has been obtained by gene manipulation, and no limitation is imposed upon the type of nucleic acid to be detected as a subject for detection.

Provided that it is a nucleic acid which has base sequences which can hybridise specifically with the nucleic acid to be detected and the nucleic acid for fixing purposes, any nucleic acid can be used for the probe nucleic acid, but oligo-nucleotides with a comparatively short nucleotide chain which can be synthesised readily with a synthesising machine are easy to use.

The nucleotide chain length of the probe nucleic acid is preferably not more than one tenth of the nucleotide chain length of the nucleic acid to be detected.

Moreover, in this invention, when labelling is carried out on the double strand part formed by the probe nucleic acid and the nucleic acid to be detected, there are no essential requirements for labelling on the probe nucleic acid itself.

For example, in the case of labelling with the niche translation method or with the labelling enzyme binding method, the labelled nucleic acid must have a nucleotide chain length greater than a certain level, but the probe nucleic acid used in the present invention does not have to satisfy any such requirement.

Hence, nucleic acids which have a short nucleotide chain length which are easily procured (prepared), and with which highly sensitive detection as described above can be achieved, can be used for the probe nucleic acid.

However, when the labelling is carried out on the double strand part formed by the nucleic acid to be detected and the probe nucleic acid in this invention, the structure or nucleotide chain length of the probe nucleic acid is preferably selected in such a way as to facilitate the labelling of the said double strand forming part.

For example, there is a method of labelling as described hereinafter in which, using one of the nucleotide chains of the double strand part formed by the probe nucleic acid and the nucleotide to be detected as a primer, the nucleotide chain is extended from its end and a labelling substance is incorporated into the extended part. When the probe nucleic acid is used as the primer, the said double strand part must have a structure such that the nucleotide chain of the nucleic acid to be detected which is hybridised with the probe nucleic acid is able to function as a template when extending the primer end. This is to say that it must have a structure where the nucleotide chain of the nucleic acid to be detected is in a single strand form in the end-extending direction of the probe nucleic acid.

Hence, with this method of labelling, in which such a probe nucleic acid is used as a primer, the structure and nucleotide chain length of the probe nucleic acid are preferably determined in consideration of the structure of the double strand part which is formed.

Moreover, depending on the particular case, the sample nucleic acid may be used as the primer.

Those structures which have a B-part which can hybridise with the nucleic acid for fixing purposes on the 5'-end, for example, and an A-part which can

hybridise with the nucleic acid to be detected on the 3'-end, and which can form a linking structure with each of the nucleic acids as shown in figure 1, for example, can be cited as probe nucleic acid structures.

Nucleic acids which have a base sequence which can hybridise with the probe nucleic acid can be used as the nucleic acids for fixing purposes provided that they can be bound to a solid phase but, as in the case of the probe nucleic acids, those which have a nucleotide chain which can be synthesised easily with a synthesiser can be used more easily. The chain length of the nucleic acid for fixing purposes is also preferably determined in consideration of the structure and nucleotide chain length of the probe nucleic acid. Moreover, in those cases where a separation treatment is carried out with the nucleic acid for fixing purposes in which the nucleic acid has been hybridised with the probe nucleic acid and the nucleic acid for fixing purposes is reused, a nucleic acid for fixing purposes which has a structure in which consideration has been given to the separation procedure may be used.

A structure into which a substance has been introduced which binds specifically and selectively with a substance which has been fixed onto a solid phase as described hereinafter can be used as the structure for fixing the nucleic acid for fixing purposes onto a solid phase. For example, avidin can be cited as a substance which can be fixed onto a solid phase and biotin can be cited as a substance which binds selectively and specifically with this and which can be bound to a nucleotide chain. Such a combination can be used provided that the two substances bind selectively and specifically and they can be introduced



into the nucleotide chain of the nucleic acid for fixing purposes and onto the solid phase.

The hybridisation of the probe nucleic acid and the nucleic acid for fixing purposes can be carried out in the usual way.

Moreover, the conditions for the hybrid-forming reaction differ according to the nucleotide chain length and the base sequences of the nucleic acid for fixing purposes, and the probe nucleic acid which are being used. The optimum conditions for the hybridisation procedure therefore should be selected appropriately in accordance with the desired result.

This hybrid-forming reaction can generally be carried out with temperature control in a hybridisation solution which contains formamide, the appropriate salts, and Denherdt solution.

Methods in which a substance which binds selectively and specifically with the specified substance for fixing purposes of the nucleic acid for fixing purposes is bound physically or chemically onto a support such as a gel or nitrocellulose, for example, and a reaction is caused to take place subsequently between the substances which bind selectively and specifically, can be used to fix the nucleic acid for fixing purpose.

In the method of this invention the probe nucleic acid is reacted with the sample and selective labelling is carried out on the hybrid which is formed as a result of the reaction.

The methods in which, when one end of the chain which forms the part of the hybrid where a double strand is formed is used as a primer and a single strand part is formed into a double strand as the end is extended, a labelling substance is included in the

extended part which is freshly produced, for example, can be used as the method for this labelling.

The above-mentioned process a) in the method of the present invention can be carried out, for example, by reacting the sample and the probe nucleic acid under the conditions required for double strand formation between the A-part of the probe nucleic acid and the nucleic acid to be detected, and then reacting the nucleic acid for fixing purposes with the reaction mixture so obtained under the conditions required for double strand formation between the B-part of the probe nucleic acid and the nucleic acid for fixing purposes.

In those cases where the nucleic acid to be detected is included in the sample a nucleic acid to be detected 1 - probe nucleic acid 2 - nucleic acid for fixing purposes 3 hybrid, as shown in figure 1, is formed by means of this procedure.

The methods in which the sample is reacted with the probe nucleic acid and the nucleic acid for fixing purposes at the same time in a hybridisation solution, the methods in which the sample and the probe nucleic acid are hybridised and the nucleic acid for fixing purposes is hybridised in the reaction mixture so obtained, and the methods in which the probe nucleic acid and the nucleic acid for fixing purposes are hybridised and the sample is hybridised in the reaction mixture so obtained, for example, can be used for the hybrid formation reaction. There are also cases in the above where the nucleic acid for fixing purposes is fixed to a solid phase.

That is to say, the probe nucleic acid and the nucleic acid for fixing purposes can be reacted first of all, and then the sample and the reaction mixture which has been obtained can be reacted.

The invention can be executed, for example, by carrying out process a) in the liquid phase, and carrying out a treatment which is required to fix the nucleic acid for fixing purposes, included in the reaction mixture which has been obtained, to a solid phase in the said reaction mixture. At this time, in those cases where a nucleic acid to be detected - probe nucleic acid - nucleic acid for fixing purposes hybrid has been formed in the said reaction mixture, this hybrid is also fixed to the solid phase via the nucleic acid for fixing purposes, and a fixed state on the solid phase 5 like that shown in figure 2, for example, is obtained.

Furthermore, the treatment for fixing the nucleic acid for fixing purposes to a solid phase can also be carried out before the reaction for forming the double strand. Actual examples of such methods include the methods where the sample and the probe nucleic acid are reacted simultaneously with a nucleic acid for fixing purposes which has been fixed to a solid phase, the methods in which the probe nucleic acid is reacted with the nucleic acid for fixing purposes which has been fixed to a solid phase and then the sample is reacted, the methods in which the sample and the probe nucleic acid are reacted and the reaction mixture is reacted with the nucleic acid for fixing purposes which has been fixed on a solid phase, and the methods in which the nucleic acid for fixing purposes and the probe nucleic acid are reacted and the nucleic acid for fixing purposes - probe nucleic acid hybrid obtained is fixed to the said phase and the sample is then reacted with the said hybrid.

The abovementioned process b) in the method of this invention can be carried out, for example, by

selectively labelling the nucleic acid to be detected - probe nucleic acid hybrid and achieving detection with a method which corresponds with the label which has been used.

Methods in which, when one chain of the double strand forming part is taken as a primer and the other chain is used as a template and the end of the chain which forms the primer is extended as mentioned earlier, a label is incorporated into the newly-formed extended part can be used for labelling.

A case where the 3'-end of the A-part of the probe nucleic acid is used as the primer is shown in figure 2.

In practical terms, for example, the methods in which a nucleotide such as dATP, dCTP, dGTP, dTTP which is required for the extension of the primer end part and the hybrid which is to be labelled is reacted in the presence of an enzyme for nucleotide chain forming purposes, and at this time one of the nucleotides which is used incorporates a label into the nucleotide chain which is being newly formed using the labelling nucleotide on the template can be used.

The labels generally used for labelling probes, for example, those which label by means of a radioactive isotope (RI) and those which label by means of a non-radioactive labelling substance (non-RI) such as an enzyme or compound which is required to induce fluorescence, luminescence or coloration, such as biotin and dinitrophenylnucleotide derivatives for example, can be used.

Various types of DNA polymerase, such as Klenow-fragments of E. coli DNA polymerase I and DNA polymerase II, and T<sub>4</sub>DNA polymerase, for example, and

reverse transcription enzymes, can be used as the enzyme for nucleotide chain forming purposes.

The labelling in this invention can be carried out in the state where the hybrid is fixed to a solid phase via the nucleic acid for fixing purposes. The time at which the labelling process is introduced is preferably after the nucleic acid to be detected and the probe nucleic acid have formed a hybrid.

Furthermore, with this method there is no part which functions as a primer and no part which can form a template for the formation of an extended part to form a new double strand part with a nucleic acid which has not formed a hybrid, and so the double strand forming reaction which incorporates the above-mentioned labelling substance does not occur.

Moreover, separation of the labelled hybrid and the label which has not been incorporated into a hybrid after the labelling reaction has been completed can be carried out using methods such as those indicated below, for example. That is to say, in a case where a hybrid has been formed between the sample and a probe nucleic acid which has been fixed to a solid phase via a nucleic acid for fixing purposes, the hybrid is also in a state where it is fixed to a solid phase. The label which has not been incorporated into the hybrid can be removed by washing in this state.

Furthermore, in a case where the reaction sequence of the sample, the probe nucleic acid and the nucleic acid for fixing purposes has been modified in any of the various ways mentioned earlier, a state where the hybrid is fixed onto a solid phase via the nucleic acid for fixing purposes is also ultimately obtained and the label which has not been incorporated into the hybrid can be subsequently separated by

carrying out a washing treatment in the same way as described above.

The detection of the label incorporated into the hybrid can be carried out, for example, using methods in which the label which has been incorporated into the hybrid in a state where the hybrid is fixed to a solid phase as shown in figure 2 is detected using a method which corresponds with the said label, and methods in which the bound part between the nucleic acid for fixing purposes and the probe nucleic acid of the hybrid which has been fixed onto a solid phase is broken and the label which has been incorporated into the nucleic acid to be detected - probe nucleic acid hybrid which has been released from the solid phase is detected using a method which corresponds with the said label.

Moreover, in the method of this invention, the solid phase onto which the nucleic acid for fixing purposes is bound, obtained on breaking the bonding part between the probe nucleic acid and the nucleic acid for bonding purposes of the hybrid which is fixed to the solid phase, can be reused repeatedly in subsequent detection reactions. If at this time a number of types of probe nucleic acid which have a common B part corresponding to the nucleic acid for fixing purposes which is being reused and different A parts corresponding to the nucleic acids to be detected are used, then the nucleic acid for fixing purposes which has been fixed to a solid phase can be reused repeatedly for the detection of different nucleic acids.

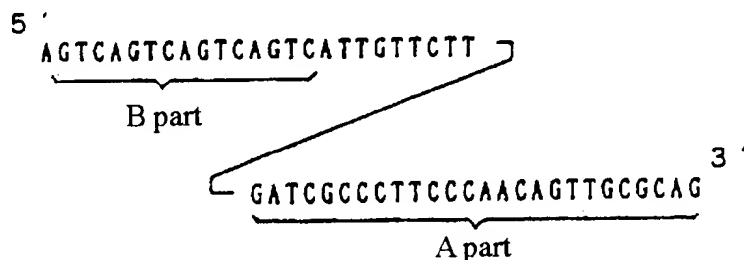
Furthermore, it is possible by measuring the amount of label incorporated into the hybrid in the

method of this invention to determine the amount of nucleic acid to be detected.

#### Illustrative Example

Detection of a gene which had part of the base sequence of the plasmid pUC19 was carried out.

An oligonucleotide, which had the structure indicated below with an A part corresponding to part of the base sequence of the plasmid pUC19, a B part which had a complementary base sequence corresponding to the nucleic acid for fixing purposes described hereinafter and an intervening base sequence which separated the A and B parts, was synthesised using DNA synthesising apparatus (Model 381A, Applied Biosystems Co.) to provide the probe nucleic acid 1.



Next, an oligonucleotide which had the base sequence indicated below was synthesised with DNA synthesising apparatus as an oligonucleotide for use in the preparation of a nucleic acid for fixing purposes.



Samples of the synthesised oligonucleotides were taken and their purity was investigated by means of electrophoresis in 20% polyacrylamide gel which contained 7M urea. The results indicated purities of over 95%, and so they were used in the following reactions without carrying out any further refinement.

Moreover, biotin was incorporated by means of the procedure indicated below into the oligonucleotide used to prepare the nucleic acid for fixing purposes which had been obtained in the way outlined above and a nucleic acid for fixing purposes was obtained.

The synthetic oligonucleotide (50  $\mu\ell$ , 50  $\mu\text{g}$ ), 100  $\mu\ell$  of liquid reagent mixture (0.46M potassium cacodilate, 0.12M Tris-OH of pH 6.9, 3.3 mM  $\text{CoCl}_2$  and 0.33 mM dithiothreitol), 40  $\mu\ell$  of 4.0 mM biotinized UTP (manufactured by the BRL Co.), 1  $\mu\ell$  of 1.0 mM dTTP, 100  $\mu\ell$  of  $\text{H}_2\text{O}$  and 15  $\mu\ell$  (about 90 units) of TdT were mixed together, and a reaction was carried out at 30°C. After 10 minutes, 4  $\mu\ell$  of 0.2M EDTA was added to the reaction system and the enzyme reaction was terminated. A phenol treatment and ethanol precipitation were carried out and the precipitate obtained was dried and then dissolved in 100  $\mu\ell$  of  $\text{H}_2\text{O}$ .

Next, using the plasmids pUC19 and pBR322, and a mixture (1 : 1) thereof, as samples, each sample was digested with EcoRI in the usual way, and the digested material obtained in this way was heat treated. The double strand DNA was converted to single strands, and the following procedure was carried out using the three types of single strand DNA mixture obtained from the samples individually.

The single strand DNA mixture (20  $\mu\text{g}$ ) was introduced into a test tube, along with 2  $\mu\text{g}$  of the nucleic acid for fixing purposes and 2  $\mu\text{g}$  of the probe nucleic acid prepared earlier. 10  $\mu\ell$  of 10X annealing solution (100 mM Tris-HCl of pH 8.0, 60 mM  $\text{MgCl}_2$ , 60 mM  $\beta$ -mercaptoethanol and 500 mM NaCl) were added and then distilled water was added to provide a total volume of



100  $\mu\text{l}$ . The solution obtained was heated to 65°C and maintained at this temperature for 10 minutes, after which it was allowed to cool slowly to room temperature over a period of about 1 hour. The reaction state at this time is illustrated schematically in figure 2.

Next, 10  $\mu\text{l}$  of 10X annealing solution, and 10  $\mu\text{l}$  each of 1 mM dATP, 1 mM dCTP and 1 mM dGTP were added to 100  $\mu\text{l}$  of the reaction liquid obtained. 100  $\mu\text{l}$  of  $\text{P}^{32}$ -TTP and distilled water were added to provide a total volume of 200  $\mu\text{l}$  to prepare a solution for labelling purposes. Klenow fragments of DNA polymerase I (5 units) were added to this solution and reacted for 1 hour with ice cooling. The state at this time is illustrated schematically in figure 3.

After reaction, the reaction liquid was mixed with a solid phase comprising agarose gel which had been activated with cyanobromide and to which avidin had been bound and then, after mixing gently for 20 minutes, the mixture was subjected to light centrifugal separation at normal temperature and the supernatant liquid was discarded. The precipitate obtained was washed twice with TE buffer solution and then the radioactivity count was measured several times using a scintillation counter. Strong counts of from  $10^6$  to  $10^7$  cpm were obtained where pUC19 or a mixture of pUC19 and pBR322 had been used, while the value for the sample of pBR322 alone did not reach double the blank value.

Furthermore, the above-mentioned precipitates were added to TE buffer solution and heated for 10 minutes at 80°C. Afterwards they were subjected to light centrifugal separation and the supernatant liquids were discarded, and then the precipitates were

washed with TE buffer solution and the radioactivities measured. The results indicated no radioactivity in the precipitates and confirmed that the part where the label had been incorporated had been released by this heat treatment.

Next, the precipitate which had been subjected to this heat treatment was reused in the same procedure as described above, and good nucleic acid fixing was achieved. Hence it was confirmed that the said precipitates had the nucleic acid for fixing purposes bound to the cyanobromide activated agarose by means of avidin-biotin bonding, and that they could be used repeatedly.

#### Effect of the Invention

Conventionally, nucleic acid fragments corresponding to the base sequence of the target gene which is being detected have been used, and it has been necessary to carry out a fixing treatment so that this can be used as a fixed probe.

Furthermore, if the type of gene to be detected is changed then new nucleic acid fragments corresponding to the gene have had to be used and another fixing treatment has been required for these fragments. Thus it has been necessary to form a corresponding fixed probe each time the gene to be detected is changed.

However, in the present invention, by using a probe for fixing purposes and a probe nucleic acid which has a sequence complementary therewith, only the probe nucleic acid need be prepared, even if the type of gene nucleic acid to be detected is changed and there is no need to carry out a fixing treatment where it is fixed to a solid phase. In this way the detection of nucleic acids can be carried out quickly and easily.

Furthermore, in this invention the separation of the hybrid into which a label has been incorporated and the label which has not been incorporated can be carried out very effectively between the solid phase and the liquid phase.

Moreover, in this invention, the nucleic acid for fixing purposes which is bound to a solid phase using an avidin - biotin bond, for example, can be reused after carrying out a probe nucleic acid releasing treatment. Hence, by using a nucleic acid for fixing purposes which has been fixed onto a solid phase, the binding treatment of the nucleic acid onto a solid phase in a new detection procedure can be omitted and the procedure is very simple.

#### **4. Brief Explanation of the Drawings**

Figure 1 is a schematic drawing which shows the structure of a nucleic acid to be detected - probe nucleic acid - nucleic acid for fixing purposes hybrid; figure 2 is a schematic drawing which shows the state where the hybrid shown in figure 1 has been bound to a solid phase; figure 3 is a schematic drawing which shows the labelling process of the fixed hybrid shown in figure 2.

- 1: Nucleic acid to be detected
- 2: Probe nucleic acid
- 3: Nucleic acid for fixing purposes
- 4: Label
- 5: Solid phase

Figure 1

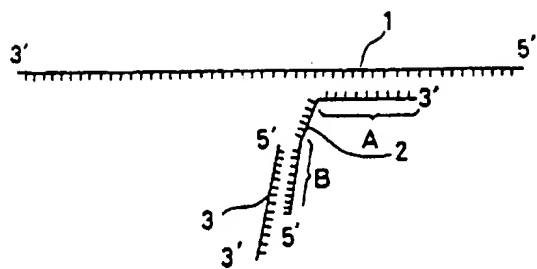


Figure 2

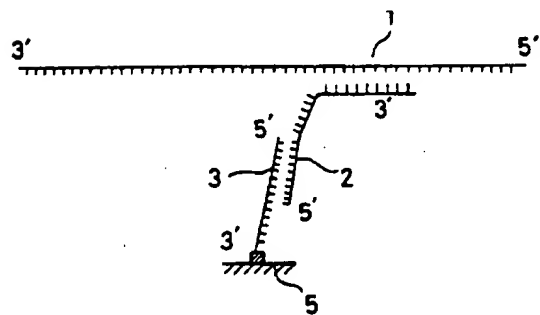
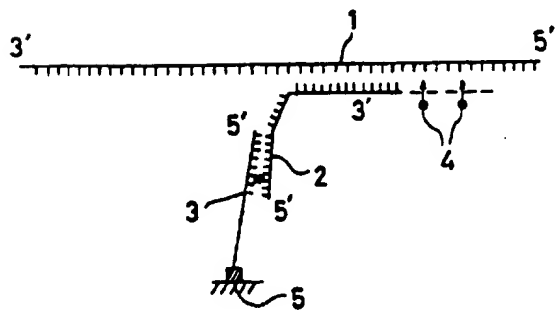


Figure 3



⑪ Int. Cl.<sup>3</sup>

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## ⑭ 発明の名称 核酸の検出方法

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## 明 細 書

## 1. 発明の名称

## 核酸の検出方法

## 2. 特許請求の範囲

1) 被検出核酸を含む試料と、

プローブ核酸と、

固相に結合された部分と、プローブ核酸とハイブリダイズする部分とを有する固定化用核酸を用い、

a) 被検出核酸・プローブ核酸・固定化核酸ハイブリッドを形成する過程と、

b) 固相に結合されたハイブリッドを標識を利用して被検出核酸・プローブ核酸ハイブリッドの形成の有無を検出する過程と

を含むことを特徴とする核酸の検出方法。

2) 被検出核酸とプローブ核酸との二本鎖形成部に選択的に標識を施す請求項1に記載の核酸の検出方法。

3) 被検出核酸、プローブ核酸及び固定化用核酸を同時に反応させる請求項1に記載の核酸の検出

方法。

4) 被検出核酸とプローブ核酸を反応させ、得られた反応混合物に固定化用核酸を反応させる請求項1に記載の核酸の検出方法。

5) プローブ核酸と固定化用核酸とを反応させ、得られた反応混合物に被検出核酸を反応させる請求項1に記載の核酸の検出方法。

6) 被検出核酸・プローブ核酸ハイブリッドを固定化用核酸より解離する過程を有する請求項1に記載の核酸の検出方法。

## 3. 発明の詳細な説明

## 〔産業上の利用分野〕

本発明は核酸のハイブリッド形成反応を利用した核酸の検出方法に関する。

## 〔従来の技術〕

試料中に検出対象としての核酸(被検出核酸)が存在するか否かを検出するための方法として種々の方法が知られている。

例えば、固相に固定した試料に標識化プローブ核酸を反応させ、固相上での被検出核酸・プロ

ブ核酸ハイブリッドの形成の有無をプローブ核酸に施した標識により検出する方法、液相中で試料とプローブ核酸を反応させ、得られた反応混合物中への被検出核酸・プローブ核酸ハイブリッドの形成の有無を検出する方法がある。

前者の方法の一例では、まず試料を電気泳動で分離し、プロットングすることによりニトロセルロースフィルターに試料の電気泳動パターンのレプリカを形成し、このレプリカに放射性プローブ核酸を反応させる。その際、試料中に被検出核酸が存在する場合には、被検出核酸と放射性プローブ核酸との間にハイブリッドが形成される。その後ハイブリダイズしなかった放射性プローブ核酸を洗浄、除去したのち、ニトロセルロースフィルター上に形成されたハイブリッドの放射性標識領域をオートラジオグラフィーなどによって検出する。

後者の方法の一例においては、溶液中で試料と放射性プローブ核酸とを反応させ、試料中に被検出核酸が含まれている場合に形成されるハイブ

ができず汎用性に欠ける。また、複数の核酸塩基配列の検出を行う場合には、それぞれの塩基配列に対応するプローブ核酸を作成し個別のカラムに固定化を行わなければならないという問題がある。

本発明はハイブリダイゼーション法を利用する従来の各種検出方法における問題点を解決するために鋭意検討した結果なされたものであり、特定の検出対象としての核酸の存在を速やかにかつ簡単に検出できる方法を提供することを目的とする。

〔課題を解決するための手段〕

本発明の検出方法は、

被検出核酸を含む試料と、

プローブ核酸と、

固相に結合された部分と、プローブ核酸とハイブリダイズする部分とを有する固定化用核酸を用い、

a) 被検出核酸・プローブ核酸・固定化用核酸ハイブリッドを形成する過程と、

リッドと未反応物とをカラム処理により分離し、形成されたハイブリッド中の放射性同位元素の標識活性を測定することで検出を行っている。

また、カラムにプローブ核酸を固定し、試料を流して反応させ、その後形成されたハイブリッドを抽出したのち1本鎖に解離し、それを集めて検出する方法もある。

〔発明が解決しようとする課題〕

前述の固相に固定した試料を用いる方法では、試料を大量に浪費したり検出操作に時間がかかるうえ、固相の調製が複雑で多くの工程を要するという欠点がある。

また、液相で試料とプローブ核酸を反応させ、生成ハイブリッドと未反応物とをカラム処理により分離する方法においては、被検出核酸のヌクレオチド鎖が比較的短い場合、カラム処理による分離精度が悪くなるという欠点がある。

また、カラムに固定したプローブ核酸を用いる方法では、カラムが特定の用途、すなわち、特定の核酸塩基配列を検出する場合にしか用いること

b) 固相に結合されたハイブリッド標識を利用して被検出核酸・プローブ核酸ハイブリッドの形成の有無を検出する過程と

固定化用核酸を利用して得る過程と

を含むことを特徴とする。

本発明は生体由来するDNAや遺伝子操作によって得られるDNAなど各種核酸の検出に利用でき、検出対象となる被検出核酸の種類は限定されない。

プローブ核酸としては、被検出核酸及び固定化用核酸と特異的にハイブリダイズできる塩基配列を有する核酸であればどのようなものでも利用できるが、合成機で手軽に合成できる比較的短いヌクレオチド鎖長のオリゴヌクレオチドが利用しやすい。

プローブ核酸のヌクレオチド鎖長は、被検出核酸のヌクレオチド鎖長の1/10以下とするのが好ましい。

なお、本発明において、プローブ核酸と被検出核酸との二本鎖形成部に標識を施す場合、プロー

ブ核酸自体に標識化に必要な要件が要求されない。

例えば、ニックトランスレーション法や標識酵素の結合法を用いる標識では、標識される核酸がある程度以上のヌクレオチド鎖長を有する必要があるが、本発明で利用するプローブ核酸にはこのような要件は要求されない。

従って、入手（調製）し易く、かつ上述のように高感度な検出を実現し得る短いヌクレオチド鎖長のものがプローブ核酸として利用できるようになる。

しかしながら、本発明において被検出核酸とプローブ核酸の二本鎖形成部に標識が施される場合、プローブ核酸の構成やヌクレオチド鎖長を、該二本鎖形成部の標識化が容易であるように選択することが望ましい。

例えば、後述するような、被検出核酸とプローブ核酸の二本鎖形成部の一方のヌクレオチド鎖をプライマーとして利用し、その末端からヌクレオチド鎖を伸展させ、その伸展部分に標識物質を取

り込ませる方法による標識化方法で、プローブ核酸をプライマーとして利用する場合、該二本鎖形成部が、プローブ核酸とハイブリダイズした被検出核酸のヌクレオチド鎖がプライマー端部の伸展の際の鋳型として機能できるように構成、すなわちプローブ核酸の末端伸展方向に被検出核酸のヌクレオチド鎖が一本鎖の状態で存在する構成を有している必要がある。

従って、このようなプローブ核酸をプライマーとして利用するこの標識化方法の場合には、プローブ核酸の構成やヌクレオチド鎖長を、形成される二本鎖形成部の構造を考慮して決定するのが望ましい。

なお、場合によっては試料核酸をプライマーとして利用するものであっても良い。

プローブ核酸の構成としては、例えば5'末端側に固定化用核酸とハイブリダイズできるB部分を、かつ3'末端側に被検出核酸とハイブリダイズできるA部分をそれぞれ有し、例えば第1図に示すような各核酸の連結構造を形成できるものを

挙げることができる。

固定化用核酸としては、プローブ核酸とハイブリダイズできる塩基配列を有し、かつ固相と結合し得る核酸であればどのようなものでも利用できるが、プローブ核酸と同様に合成機で手軽に合成できるヌクレオチド鎖を有するものが利用し易い。この固定化用核酸の鎖長もプローブ核酸の構成やヌクレオチド鎖長を考慮して決定するのが望ましい。なお、プローブ核酸とハイブリダイズした固定化用核酸に解離処理を行い固定化用核酸を再利用したい場合は、解離操作を考慮した構成を有する固定化用核酸を用いるとよい。

固定化用核酸の固相への固定のための構成としては、後述する固相に固定化されている物質と選択特異的に結合する物質を導入した構成が利用できる。例えば、固相に固定化されている物質としてアビジン、それに選択特異的に結合し、かつヌクレオチド鎖に結合可能な物質としてビオチンがあげられるが、この両物質については選択特異的に結合し、かつ固定化用核酸のヌクレオチド鎖、

固相に導入できるものであれば、どのようなものでも利用可能である。

試料と、プローブ核酸及び固定化用核酸とのハイブリダイゼーションは、常法に従って行なうことができる。

なお、ハイブリッド形成反応の条件は、用いられるプローブ核酸、固定化用核酸の有するヌクレオチド鎖長や塩基配列などによって異なるので、ハイブリダイゼーションにおける操作条件は所望とする目的に応じて最適条件を適宜選択すると良い。

このハイブリッド形成反応は、一般的には、ホルムアミド、適当な塩及びDenhardt溶液を含むハイブリダイゼーション溶液中で、温度をコントロールして行うことができる。

固定化用核酸の固定化には、固定化用核酸の有する固定用の特定物質に選択特異的に結合する物質を各種ゲル、ニトロセルロース等の担体に物理的あるいは化学的に結合させ、その後選択特異的に結合する物質間に反応を生じさせる方法が利用

できる。

本発明の方法においては、試料とプローブ核酸を反応させ、その結果形成されたハイブリッドに選択的に標識が施される。

この標識化の方法としては、例えばハイブリッドの二本鎖形成部を構成する鎖の一方をプライマーとして利用し、その末端を伸展させて1本鎖部分を2本鎖化する際に、その新たに合成される伸展部分に標識物質を取り込ませる方法等が利用できる。

本発明の方法の上記過程aは、例えば試料とプローブ核酸とをプローブ核酸のA部分と被検出核酸とでの二本鎖形成に必要な条件下で反応させた後、得られた反応混合物に固定化用核酸をプローブ核酸のB部分と固定化用核酸とでの二本鎖形成に必要な条件下で反応させることにより行なうことができる。

この操作により、試料中に被検出核酸が含まれている場合には、例えば第1図に示すような被検出核酸1・プローブ核酸2・固定化用核酸3ハイ

ブリッドも固定化用核酸を介して固相に固定され、例えば第2図のような固定相5への固定状態が得られる。

また、固定化用核酸の固相への固定のための処理を、二本鎖形成のための反応の前に導入することにより行なうこともできる。その具体的な方法としては、固相に固定された固定化用核酸に、試料及びプローブ核酸を同時に反応させる方法、固相に固定された固定化用核酸に、プローブ核酸を反応させ次いで試料を反応させる方法、試料とプローブ核酸を反応させ、得られた反応混合物を固相に固定された固定化用核酸と反応させる方法、固定化用核酸とプローブ核酸を反応させ、得られた固定化用核酸-プローブ核酸ハイブリッドを固相に固定した後、該ハイブリッドに試料を反応させる方法がある。

本発明の方法における上記過程bは、例えば被検出核酸とプローブ核酸とのハイブリッドに選択的に標識を施し、それを用いた標識に応じた方法で検出することにより行なうことができる。

ブリッドが形成される。

このハイブリッド形成反応には、試料とプローブ核酸及び固定化用核酸とを同時にハイブリダイゼーション溶液中で反応させる方法、試料とプローブ核酸とをハイブリダイゼーションさせ、得られた反応混合物に固定化用核酸をハイブリダイゼーションさせる方法、プローブ核酸と固定化用核酸をハイブリダイゼーションさせ、得られた反応混合物に試料をハイブリダイゼーションさせる方法等が利用できる。上記において、固定化用核酸は固相に固定化されている場合もある。

すなわち、まずプローブ核酸と固定化用核酸とを反応させ、次いで得られた反応混合物と試料とを反応させても良い。

本発明は、例えば過程aを液相中で行ない、得られた反応混合物に該反応混合物に含まれる固定化用核酸の固相への固定に必要な処理を行なうことにより行なうことができる。この際、該反応混合物に被検出核酸・プローブ核酸・固定化用核酸ハイブリッドが形成されている場合には、このハ

この標識化には、前述したように二本鎖形成部の一方の鎖をプライマーとし、他の鎖を鋳型として利用して、プライマーとなる鎖の末端を伸展させる際に、新たに形成させる伸展部に標識を取り込ませる方法が利用できる。

第2図にプローブ核酸のA部分を構成する3'末端部をプライマーとして利用する場合を示す。

具体的には、例えば、プライマー末端の伸展に必要なdATP、dCTP、dGTP、dTTPなどのヌクレオチドと標識化すべきハイブリッドとをヌクレオチド鎖形成用の酵素の存在下で反応させ、その際に用いるヌクレオチドの1を鋳型に標識化ヌクレオチドを用いて、新たに形成されるヌクレオチド鎖に標識を取り込ませる方法等を利用できる。

この標識化ヌクレオチドとしては、一般にプローブの標識に利用されている、例えば放射性同位元素(RI)により標識化されたもの、例えばビオチン、ジニトロフェニルヌクレオチド誘導体等の蛍光、発光または発色を誘発するのに必要な



酵素や化合物などの非放射性標識物質 (non RI) で標識化されたものなどが利用できる。

ヌクレオチド鎖形成用の酵素としては、大腸菌 DNA ポリメラーゼ I、DNA ポリメラーゼ I のクレノー断片、T. DNA ポリメラーゼ等の各種 DNA のポリメラーゼや逆転写酵素などが利用できる。

本発明における標識化は、ハイブリッドが固定化用核酸を介して固相に固定化された状態で行なうことができる。標識化過程を導入する時期としては、被検出核酸とプローブ核酸とがハイブリッドを形成した後に行なうのがよい。

また、この方法によれば、ハイブリッドを形成していない核酸には、新たな二本鎖部分形成のためのプライマーとして機能する部分及び伸展部分形成用の鋳型となる部分が存在しないので、上記の標識物質を取り込む二本鎖化反応が生じない。

なお、標識化の反応終了後に、標識化されたハイブリッドと、ハイブリッドに取り込まれなかった標識との分離は、例えば以下のような方法によ

り行なうことができる。

なお、本発明の方法においては、固相に固定されたハイブリッドのプローブ核酸と固定化用核酸との結合部を解離させて得られる固定化用核酸が結合した固相は、次の検出反応に繰返し再利用可能である。その際、B部分を再利用する固定化用核酸に対して共通に形成し、A部分を被検出核酸に応じて異ならせた複数種のプローブ核酸を用いれば、固相に固定された固定化用核酸を異なる被検出核酸の検出に繰返し再利用できる。

また、本発明の方法において、ハイブリッドに取り込まれた標識の量を測定することにより、被検出核酸の定量を行なうことができる。

#### 【実施例】

プラスミド pUC19 の塩基配列の一部をもつ遺伝子検出を行った。

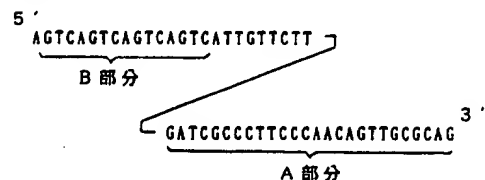
プラスミド pUC19 の塩基配列の一部に対応する A 部分と後に述べる固定化用核酸に対して相補的な塩基配列をもつ B 部分、及びその間に A と B との距離を保つ塩基配列をもった下記の構成の

り行なうことができる。すなわち固定化用核酸を介して固相に固定されたプローブ核酸と試料の間にハイブリッドが形成された場合には、ハイブリッドも固相に固定化されている状態となる。その状態で洗浄し、ハイブリッドに取り込まれなかった標識を洗い出して除去する。

また、試料、プローブ核酸及び固定化用核酸の反応順を前述のように種々変更した場合でも、最終的に固定化用核酸を介したハイブリッドの固相への固定化状態を得た後、上述と同様の洗浄処理を行なってハイブリッドに取り込まれなかった標識を分離することができる。

ハイブリッドに取り込まれた標識の検出は、例えば第 2 図に示すように固相に固定化された状態のハイブリッドに取り込まれている標識を、該標識に応じた方法で検出する方法、固相に固定化されたハイブリッドの固定化用核酸とプローブ核酸との結合部を解離させ、固相から離された被検出核酸・プローブ核酸ハイブリッドに取り込まれている標識を該標識に応じた方法で検出方法等によ

オリゴヌクレオチドを DNA 合成装置 (Applied Biosystems 社、381 A 型) により合成しプローブ核酸 1 とした。



次に固定化用核酸調製用オリゴヌクレオチドとして、下記の塩基配列を有するオリゴヌクレオチドを DNA 合成装置で合成した。



これらの合成されたオリゴヌクレオチドの一部をサンプリングし、7 M 尿素を含む 20 % ポリアクリルアミドゲル電気泳動によりその純度を調べた。その結果、95 % 以上の純度であったので、それ以上の精製を行わずに以下の反応に用いることにした。

更に、上述のようにして得た固定化用核酸調製

用オリゴヌクレオチドに以下の操作によりビオチンを導入して固定化用核酸を得た。

合成オリゴヌクレオチド  $50\mu\text{g}$  ( $50\mu\text{g}$ )、混合試薬液 ( $0.46\text{M}$  カコシル酸カリウム、 $0.12\text{M}$  Tris-OH pH6.9、 $3.3\text{mM}$   $\text{CoCl}_2$ 、 $0.33\text{mM}$  ジチオスレイトール)  $100\mu\text{g}$ 、 $4.0\text{mM}$  ビオチン化UTP (BRL社製)  $40\mu\text{g}$ 、 $1.0\text{mM}$  dTTP  $1\mu\text{g}$ 、 $\text{H}_2\text{O}$   $100\mu\text{g}$  および TdT  $15\mu\text{g}$  (約90 unit) を混合し、 $30^\circ\text{C}$  で反応を行なった。10分間経過後、 $0.2\text{M}$  EDTA  $4\mu\text{g}$  を反応系に添加し、酵素反応を停止させ、更にフェノール処理、エタノール沈殿を行い得られた沈殿物を乾燥後  $\text{H}_2\text{O}$   $100\mu\text{g}$  に溶解した。

次に、試料としてプラスミド pUC19、pBR322 及びこれらの混合物 (1:1) を用意し、各試料を常法に従い EcoRI で消化してから得られた消化物を加熱処理して、二本鎖DNAを一本鎖化し、各試料から得られた3種の一本鎖DNA混合物を個々に用いて以下の操作を行なっ

3図である。

反応後、この反応液をアガロースゲルを臭化シアンで活性化しアビジンを結合した固相と混合し、20分間ゆっくり混和させた後、常温で軽く遠心し上清を廃棄した。得られた沈殿物をTE緩衝液で2度洗浄した後その放射線の計数をシンチレーションカウンターで数度測定したところ pUC19 及び pUC19 と pBR322 の混合物を用いた場合は  $10^{-7}\text{cps}$  の強度が計数され、pBR322 のみの試料におけるその値はバックグラウンドの2倍に満たなかった。

また、上記沈殿物をTE緩衝液に加え、 $80^\circ\text{C}$  で10分間加熱を行なった後すばやく遠心し上清を廃棄した後、沈殿物をTE緩衝液で洗浄して放射活性を測定した。その結果、沈殿物中に放射活性は計量されず、この加熱処理により標識を取り込んだ部分が解離されたことが確認された。

次に、この加熱処理後に得られた沈殿物を、上述と同様の操作に再利用したところ、良好な核酸の操作が行なえた。従って、該沈殿物はアビジン

た。

一本鎖DNA混合物  $20\mu\text{g}$  と、先に調製した固定化用核酸  $2\mu\text{g}$  及びプローブ核酸  $2\mu\text{g}$  を試験管に入れ  $10\times$  アニリング溶液 ( $100\text{mM}$  Tris-HCl pH8.0、 $60\text{mM}$   $\text{MgCl}_2$ 、 $60\text{mM}$   $\beta$ -メルカプトエタノール、 $500\text{mM}$  NaCl) を  $10\mu\text{g}$  を加え、更に蒸留水を加え全体が  $100\mu\text{g}$  になるように調製した。得られた溶液を  $65^\circ\text{C}$  まで加熱し、10分間その温度を保った後約1時間かけてゆっくり室温まで冷ました。この時の反応状態を模式的に示したのが第2図である。

次に、得られた反応液  $100\mu\text{g}$  に  $10\times$  アニリング溶液  $10\mu\text{g}$ 、 $1\text{mM}$  dATP、 $1\text{mM}$  dCTP 及び  $1\text{mM}$  dGTP を各  $10\mu\text{g}$  加えた後、 $\text{p}^{32}\text{TTP}$   $100\mu\text{g}$  を添加し、さらに蒸留水を加え全液量を  $200\mu\text{g}$  として標識化用の溶液を調製した。この溶液にDNAポリメラーゼIのKlenow断片を5単位加入氷冷下で1時間反応させた。この時の状態を模式的に示したのが第

ビオチン結合により固定化用核酸が臭化シアン活性化アガロースに結合したものであり、繰返し再利用が可能であることが確認された。

#### 〔発明の効果〕

従来、検出を行なう目的遺伝子の塩基配列に応じて核酸断片を用意し、これを固定化プローブとして用いるために、固定化の処理を施す必要があった。

また、検出したい遺伝子の種類が変われば、それに従って新たな核酸断片を用意し、なおかつ、これに固定化の処理が必要となる。つまり検出する遺伝子が変わるたびに、それに応じて固定化プローブを形成する必要がある。

しかし本発明では固定化用プローブとその一部と相補的配列をもつプローブ核酸を用いることによって検出したい遺伝子核酸の種類が変わってもプローブ核酸のみを作成すればよく、固相への固定化の処理を行なう必要が全くないので、迅速かつ簡便な核酸の検出を行なうことができる。

また、本発明においては標識を取り込んだハイ

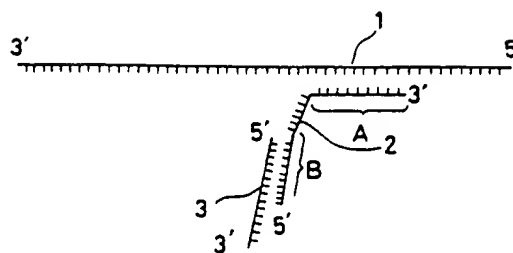
ブリッドと取り込まれなかった標識との分離が固相・液相間で精度良く行なわれるので、精度良い検出操作が可能となる。

更に、本発明においては、アビジン-ビオチン結合等を利用して固相に結合させた固定化用核酸は、プローブ核酸との解離処理を行なうことにより再利用可能である。従って、この固相に結合された固定化用核酸を利用することにより、新たな検出操作において化学的反応等を利用した固相への核酸の結合処理を省略でき、操作が極めて簡易化される。

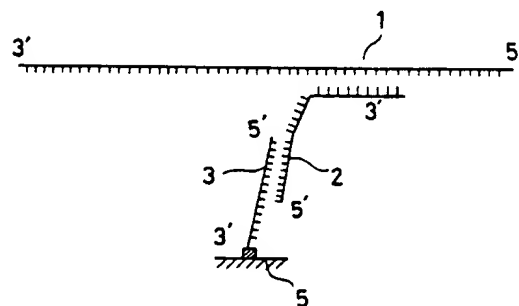
#### 4. 図面の簡単な説明

第1図は被検出核酸・プローブ核酸・固定化用核酸ハイブリッドの構成を示す模式図、第2図は第1図で示したハイブリッドを固相に固定した状態を示す模式図、第3図は第2図で示した固定化ハイブリッド標識化の過程を示す模式図である。

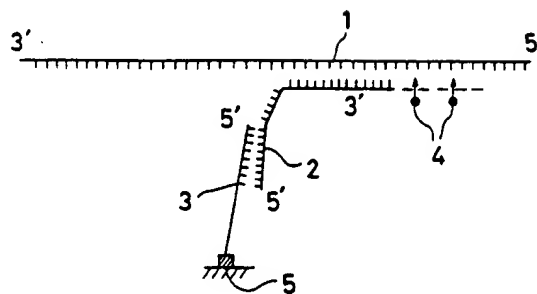
- 1 : 被検出核酸                      2 : プローブ核酸
- 3 : 固定化用核酸                  4 : 標識
- 5 : 固相



第 1 図



第 2 図



第 3 図